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Comparison of independent and combined effects of the neurotensin receptor agonist, JMV-449, and incretin mimetics on pancreatic islet function, glucose homeostasis and appetite control

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ABSTRACT

Background: Neurotensin receptor activation augments the biosctivity of glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). JMV-449, a C-terminal neurotensin-like fragment with a reduced peptide bond, represents a neurotensin receptor agonist.
Methods: The present study assessed the actions of JMV-449 on pancreatic beta-cells alone, and in combination with GIP and GLP-1. Further studies examined the impact of JMV-449 and incretin mimetics on glucose homeostasis and appetite control in mice.
Results: JMV-449 was resistant to plasma enzyme degradation and induced noticeable dose-dependent insulin-releasing actions in BRIN-BD11 beta-cells. In combination with either GIP or GLP-1, JMV-449 augmented ($P < 0.05$) the insulinotropic actions of both hormones, as well as enhancing ($P < 0.001$) insulin secretory activity of both incretin peptides. JMV-449 also increased beta-cell proliferation and induced significant benefits on beta-cell survival in response to cytokine-induced apoptosis. JMV-449 (25 nmol/kg) inhibited ($P < 0.05$–$P < 0.001$) food intake in overnight fasted lean mice, and enhanced ($P < 0.01$) the appetite supressing effects of an enzymatically stable GLP-1 mimetic. When injected co-jointly with glucose, JMV-449 evoked glucose lowering actions, but more interestingly significantly augmented ($P < 0.05$) the glucose lowering effects of established long-acting GIP and GLP-1 receptor mimetics. In terms of glucose-induced insulin secretion, only GIP receptor signalling was associated with increases in insulin concentrations, and this was not enhanced by JMV-449.
Conclusion: JMV-449 is a neurotensin receptor agonist that positively augments key aspects of the biological action profile of GIP and GLP-1.
General significance: These observations emphasise the, yet untapped, therapeutic potential of combined neurotensin and incretin receptor signalling for diabetes.

1. Introduction

Neurotensin was originally considered as an anorexigenic neuropeptide [1], with recent studies showing that this peptide is co-expressed in enteroendocrine L-cells alongside glucagon-like peptide-1 (GLP-1) and Peptide YY (PYY), and secreted into the bloodstream following nutrient ingestion [2]. Furthermore, neurotensin regulates metabolism in a similar manner to GLP-1 and PYY, with additional important interactions between all three hormones in terms of blood glucose and appetite regulation [2]. Recently, a stable neurotensin analogue has been shown to synergise with the clinically approved GLP-1 mimetic, liraglutide, to combat obesity and metabolic dysregulation in high fat fed mice [3]. In harmony with these observations, the insulinotropic bioactivity of the sister incretin hormone of GLP-1, glucose-dependent insulinotropic polypeptide (GIP), is enhanced by a peptide hormone called xenin [4–6]. Xenin is a peptide co-expressed and released with GIP from enteroendocrine K-cells following nutrient ingestion [7], with a suggestion that it acts as a neurotensin receptor agonist [8]. Interestingly, xenin and neurotensin share significant amino acid sequence homology [7,9], with the C-terminal hexapeptide sequence of both peptides, namely neurotensin (8–13) and xenin (20–25), known to function as biologically active fragment peptides [6,10].

Taken together, it appears that the biological activity of both incretin

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hormones could be enhanced through concurrent administration of neurotensin related peptides, which would have particular importance in the diabetes clinic. Indeed, the intracellular signalling pathways stimulated by GIP and GLP-1 receptor activation within pancreatic beta-cells are very similar, in that both are initially mediated by activation of a specific G-protein coupled receptor with subsequent increases of intracellular cAMP [11]. This results in comparable benefits on beta-cell function, growth and survival, but some variations in downstream signalling may account for small efficacy differences between the two peptides [11]. Unfortunately, a significant hindrance to the therapeutic applicability of neurotensin peptides relates to a short biological half-life [12]. However, metabolic stability of neurotensin can be improved through appropriate structural modification [10]. As such, introduction of a reduced peptide bond, CH_2NH, between Lys^8 and Lys^9 in neurotensin(8–13)-like peptide, generates an enzymatically stable and biologically active peptide that acts as a powerful neurotensin receptor agonist [10]. Similarly, enhanced stability and bioactivity of the C-terminal hexapeptide of xenin has also been demonstrated through introduction of a reduced pseudopeptide bond between the two N-terminal amino acid residues, to yield Ψ-xenin-6 [13]. More interestingly, Craig and colleagues have recently confirmed that Ψ-xenin-6 significantly augments the bioactivity of GIP [6]. Furthermore, when Ψ-xenin-6 was administered to high fat fed mice in combination with the dipeptidyl peptidase-4 (DPP-4) inhibitor drug sitagliptin, clear additive antidiabetic effects were noted [14]. Together, these observations confirm the applicability of metabolically stable C-terminal hexapeptides of either neurotensin or xenin. Since the receptor activation profile of xenin and related fragment peptides is not yet fully elucidated [7], neurotensin-based peptides may offer a more therapeutically attractive option to augment the biological action of incretin hormones and improve their antidiabetic efficacy.

In this regard, JMV-449 is a recognised biologically active neurotensin fragment peptide, with a reduced pseudopeptide CH_2NH bond between Lys^8 and Lys^9 [10]. In the current study, we initially aimed to characterise the effects of JMV-449 at the level of the pancreatic beta-cell. Further studies examined the ability of JMV-449 to positively interact with GIP and GLP-1 in terms of both *in vitro* pancreatic beta-cell induced insulin secretion, as well as glucose homeostasis, insulin secretion and appetite control in mice.

2. Materials and methods

2.1. Peptide synthesis

JMV-449 was obtained from Bio-Techne Ltd. at 99.4% purity (Abingdon, United Kingdom). Native GIP and GLP-1 as well as (D-Ala^2) GIP and exendin-4 were purchased from Synpeptide (Shanghai, China) at >95% purity. Before experimentation, all peptides were characterised in-house using reverse-phase HPLC and MALDI-TOF MS, as previously described [15].

2.2. In vitro insulin secretion

In vitro insulin releasing activity of test peptides was assessed in rodent BRIN-BD11 beta-cells, cultured and maintained as previously described [16]. For insulin secretory studies, BRIN-BD11 cells were seeded into 24-well plates (150,000 cells per well) and allowed to attach overnight at 37 °C. Prior to tests, cells were pre-incubated in Krebs–Ringer bicarbonate buffer (KRBB) (pH 7.4) supplemented with 0.5% (*w/v*) BSA and 1.1 mM glucose (40 min; 37 °C). Cells were incubated (20 min) with JMV-449 alone (10^{-12}–10^{-6} M), or in combination with GIP (10^{-12}–10^{-6} M) or GLP-1 (10^{-12}–10^{-6} M), as well as combination of all three test peptides. All samples were stored at −20 °C prior to assessment of insulin concentrations by an in-house RIA [17].

2.3. In vitro beta-cell proliferation and apoptosis

BRIN-BD11 beta-cells were used to investigate effects of JMV-449 (10^{-8} and 10^{-6} M) on beta-cell proliferation and protection against cytokine-induced apoptosis. GLP-1 was employed as a positive control for all studies. Ki-67 immunostaining was used to assess effects on proliferation. Briefly, cells were seeded onto coverslips (40,000 cells per coverslip) and cultured overnight (18 h;37 °C), in the presence of test peptides (10^{-8} and 10^{-6} M). Cells were then washed with PBS, and fixed using 4% paraformaldehyde. Following antigen retrieval with citrate buffer (90 °C for 20 min), tissues were blocked using 1.1% BSA for 30 min. Cells were then incubated with Ki-67 primary antibody (1:500; Abcam, ab15580), followed by Alexa Fluor® 488 secondary antibody (1:400, Invitrogen, A-11008). Coverslips were washed with PBS, mounted on slides for viewing using a fluorescent microscope (Olympus System Microscope) and photographed by DP70 camera adapter system. Proliferation frequency was expressed as percentage of total cells analysed. For analysis of the ability of JMV-449 to protect against cytokine-induced apoptosis, cells were seeded as above. However, cells were also exposed to a cytokine-cocktail (IL-1β 100 U/mL, IFN-γ 20 U/mL, TNF-α 200 U/mL, all purchased from Sigma-Aldrich) in the presence or absence of test peptides (10^{-8} and 10^{-6} M) for 18 h, with hydrogen peroxide as an additional control. TUNEL staining (Roche Diagnostics Ltd., UK) was employed to quantify beta-cell apoptosis, as previously described [18]. Apoptosis was expressed as percentage of total cells analysed. Approximately 150 cells were analysed per group.

2.4. Animals

Animal studies were carried out using male C57BL/6 mice (10 month old, Envigo Ltd., UK), all housed individually in an air-conditioned room at 22 ± 2 °C with a 12 h light: 12 h dark cycle. Animals were maintained on standard rodent chow diet (10% fat, 30% protein and 60% carbohydrate, Trouw Nutrition, UK) with *ad libitum* access to diet and water. All animal experiments were carried out in accordance with the UK Animal Scientific Procedures Act 1986 and approved by Ulster University Animal Welfare and Ethical Review Body (AWERB).

2.5. Acute effects on food intake, glucose tolerance and insulin secretion in mice

For *in vivo* studies, JMV-449 as well as fully characterised enzymatically stable forms of GIP and GLP-1 were employed, namely (D-Ala^2) GIP and exendin-4 [19,20]. For assessment of appetite control, cumulative food intake was assessed in overnight fasted (18 h) mice following i.p. injection of saline vehicle (0.9% *w/v* NaCl) or test peptide(s) (at either 2.5 or 25 nmol/kg bw, as appropriate), with food intake measured at 30 min intervals for 180 min. For assessment of glucose tolerance, blood glucose and plasma insulin concentrations were determined immediately prior to and 15, 30, 60 and 105 min after i.p. injection of glucose alone (18 mmol/kg bw) or in combination with test peptide(s) (each at 25 nmol/kg bw) in 18 h fasted mice.

2.6. Biochemical analysis

Blood samples were collected from the cut tip on the tail vein of conscious mice into chilled fluoride/heparin glucose micro-centrifuge tubes (Sarstedt, Numbrecht, Germany). Blood glucose was measured directly using a Contour blood glucose meter. For plasma insulin analysis, blood samples were collected into chilled fluoride/heparin glucose micro-centrifuge tubes (Sarstedt, Numbrecht, Germany) and immediately centrifuged using a Beckman microcentrifuge (Beckman Instruments, Galway, Ireland) for 1 min at 13,000*g* and stored at −20 °C prior to insulin RIA [17].

2.7. Statistical analysis

Statistical analysis was completed using GraphPad PRISM (Version 5). Results are expressed as means ± SEM and data compared using repeated measures ANOVA followed by Student-Newman-Keuls *post-hoc* test. Unpaired Student *t*-test was used where appropriate. Incremental plasma insulin and glucose area under the curve (AUC) were calculated using the trapezoidal rule with appropriate baseline subtraction. Groups of data were considered significant if $P < 0.05$.

3. Results

3.1. Peptide characterisation and plasma enzyme stability

MS analysis confirmed identity of JMV-449 (H-LYS-$^{\Psi}$-LYS-PRO-TYR-ILE-LEU-OH), with theoretical and experimental masses differing by less than 0.5 Da (Table 1). The HPLC profile for JMV-449 confirmed 99.4% purity as stated by the manufacturer (data not shown). When incubated with murine plasma, JMV-449 remained 100% intact up to 8 h (Table 1).

3.2. In vitro insulin secretory actions

In BRIN-BD11 cells, JMV-449 displayed significant ($P < 0.05$ to $P < 0.01$) dose-dependent insulin secretory activity at 5.6 mM glucose when compared to control cultures (Fig. 1A). At 16.7 mM glucose, significant ($P < 0.01$) insulin secretory actions were displayed only at concentrations of 10^{-6} M (Fig. 1B). Importantly, as previously observed, there was a clear glucose-dependent increase of insulin secretion in our BRIN BD11 cell line, with an insulin output of 0.3 ± 0.1 and 0.8 ± 0.1 ng/mL at 5.6 and 16.7 mM glucose, respectively. When JMV-449 was incubated in combination with GIP, significant ($P < 0.01$ to $P < 0.001$) dose-dependent insulinotropic actions were exhibited at 10^{-10} M and above, with combination treatment being significantly ($P < 0.05$) more effective than GIP alone at 10^{-6} M (Fig. 1C). A similar insulin secretory profile for JMV-449 and GIP was evident at 16.7 mM glucose, although JMV-449 was unable to augment GIP-induced insulin secretion under hyperglycaemic conditions (Fig. 1D). Largely similar insulinotropic benefits were observed when JMV-449 was co-incubated with GLP-1 at 5.6 and 16.7 mM glucose (Fig. 1E,F). However, JMV-449 was able to significantly ($P < 0.05$) augment the insulinotropic actions of 10^{-6} M GLP-1 at 5.6 mM glucose (Fig. 1E). Interestingly, when JMV-449 was co-incubated with both GIP and GLP-1, there was a significant ($P < 0.001$) augmentation of insulin secretion at 5.6 and 16.7 mM glucose (Fig. 1G, H). Moreover, combined JMV-449, GIP and GLP-1 treatment at 5.6 mM glucose was significantly ($P < 0.001$) more effective than either GIP or GLP-1 alone (Fig. 1G).

3.3. Beta-cell proliferation and protection against apoptosis

Similar to GLP-1, JMV-449 significantly ($P < 0.001$) augmented BRIN-BD11 beta-cell proliferation at concentrations of 10^{-8} and 10^{-6} M (Fig. 2A). In relation to protection against cytokine-induced apoptosis, JMV-449 significantly ($P < 0.05$ and $P < 0.001$) augmented BRIN-BD11 beta-cell survival in response to cytokine-induced apoptosis at 10^{-8} and 10^{-6} M (Fig. 2B), similar to the protective nature of GLP-1. Indeed, at concentrations of 10^{-6} M, both peptides fully reversed the detrimental effect of cytokine culture on beta-cell apoptosis (Fig. 2B) Representative images of Ki-67 and TUNEL staining for each incubation are shown in Fig. 2C&D.

3.4. Acute in vivo effects on food intake

JMV-449, (D-Ala^2)GIP, and exendin-4, induced a significant ($P < 0.05$ and $P < 0.01$) decrease in food intake in overnight fasted lean mice (Fig. 3A,B). JMV-449 was unable to augment the appetite suppressive actions of (D-Ala^2)GIP or exendin-4 at a dose 25 nmol/kg (Fig. 3A,B). At the same peptide dose, combined injection of (D-Ala^2)GIP and exendin-4 had a profound effect to reduce food intake, but this effect was not altered by co-injection with JMV-449 (Fig. 3C). However, when exendin-4 was administered at a reduced dose of 2.5 nmol/kg, JMV-446 was able to augment ($P < 0.01$) the appetite suppressive actions of this peptide (Fig. 3D). Following combined administration of 25 nmol/kg (D-Ala^2)GIP and JMV-449, together with 2.5 nmol/kg exendin-4, there was a significant reduction ($P < 0.01$) of food intake at 15 min post-injection when compared to (D-Ala^2)GIP plus exendin-4 injection alone (Fig. 3E).

3.5. Acute in vivo effects on glucose tolerance and insulin secretion

Administration JMV-449 in combination with glucose to lean mice resulted in significantly ($P < 0.05$ to $P < 0.01$) decreased individual plasma glucose levels at 15 and 30 min post-injection when compared to glucose alone control, but this did not translate to significant reductions in 0–105 min AUC values (Table 2). However, plasma insulin AUC was significantly ($P < 0.05$) increased by JMV-449, despite lack of effect on individual glucose-stimulated insulin concentrations (Table 3). As expected, (D-Ala^2)GIP evoked significant glucose homeostatic and insulin secretory actions in mice (Tables 2 & 3). Notably, the overall glucose-lowering effect of (D-Ala^2)GIP was significantly ($P < 0.05$) enhanced by JMV-449 (Table 2). An essentially similar biological action profile was noted when JMV-449 was co-administered with exendin-4, with slightly less prominent effects on glucose-induced insulin secretion (Tables 2 & 3). As such, both compounds had impressive glucose-lowering actions (Table 2), with JMV-449 significantly enhancing the glucose homeostatic action of exendin-4 (Table 2). Interestingly, whilst combined administration of (D-Ala^2)GIP and exendin-4 exerted pronounced effects to reduce blood glucose levels (Table 2), this was not associated with obvious benefits on insulin secretion (Table 3). JMV-449 was unable to enhance the marked positive effects of combined (D-Ala^2)GIP and exendin-4 injection (Tables 2 & 3).

4. Discussion

In the present study, we aimed to characterise the insulinotropic and metabolic actions of the neurotensin receptor agonist, JMV-449 [10], as well as examining positive interactions between JMV-449 and incretin hormones. As such, there is suggestions that the antidiabetic efficacy of

Table 1
Amino acid sequence, MALDI-TOF MS analysis and enzymatic stability of JMV-449.

Peptide	Sequence	Theoretical molecular mass (Da)	Experimental molecular mass (Da)	Percentage intact peptide remaining (%)			
				2 h	4 h	6 h	8 h
JMV-449	H-LYS-$^{\Psi}$-LYS-PRO-TYR-ILE-LEU-OH	747.0	747.2	100	100	100	100
Neurotensin	GLU-LEU-TYR-GLU-ASN-LYS-PRO-ARG-ARG-PRO-TYR-ILE-LEU-OH	1672.9	*ND*	*ND*	*ND*	*ND*	*ND*

Molecular mass of JMV-449 was determined using MALDI-TOF MS. Peptide stability was assessed following 0, 2, 4, 6 and 8 h incubation in murine plasma. Degradation products were separated using HPLC, analysed by MS, and percentage degradation calculated from peak areas. The amino acid sequence of neurotensin is included for comparative purposes. *ND*, not determined.

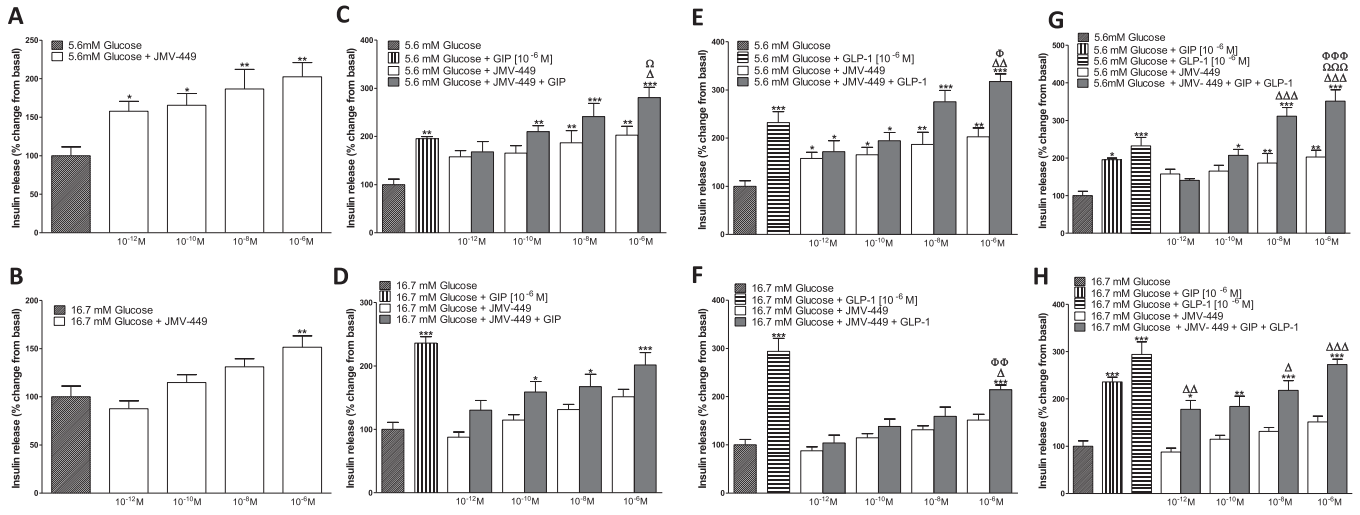

Fig. 1. Acute effects of JMV-449 alone, and in combination with GIP or GLP-1, on insulin release from BRIN-BD11 cells.
BRIN-BD11 cells were incubated (20 min) with a range of concentrations (10^{-12} to 10^{-6} M) of JMV-449 alone (A,B) or in combination with GIP (C,D), GLP-1 (E,F) or together with both incretin peptides (G,H) in the presence of (A,C,E,G) 5.6 or (B,D,F,H) 16.7 mM glucose. Insulin release was measured using radioimmunoassay. Values represent means ± SEM ($n = 8$ for insulin). *P < 0.05, **P < 0.01 and ***$P < 0.001$ compared to respective glucose controls. $^{\Delta}$P < 0.05, $^{\Delta\Delta}$P < 0.01 and $^{\Delta\Delta\Delta}$P < 0.001 compared to respective JMV-449 alone. $^{\Omega}$P < 0.05 and $^{\Omega\Omega\Omega}$ P < 0.001 compared to 10^{-6} M GIP. $^{\Phi}$P < 0.05, $^{\Phi\Phi}$P < 0.01 and $^{\Phi\Phi\Phi}$P < 0.001 compared to 10^{-6} M GLP-1.

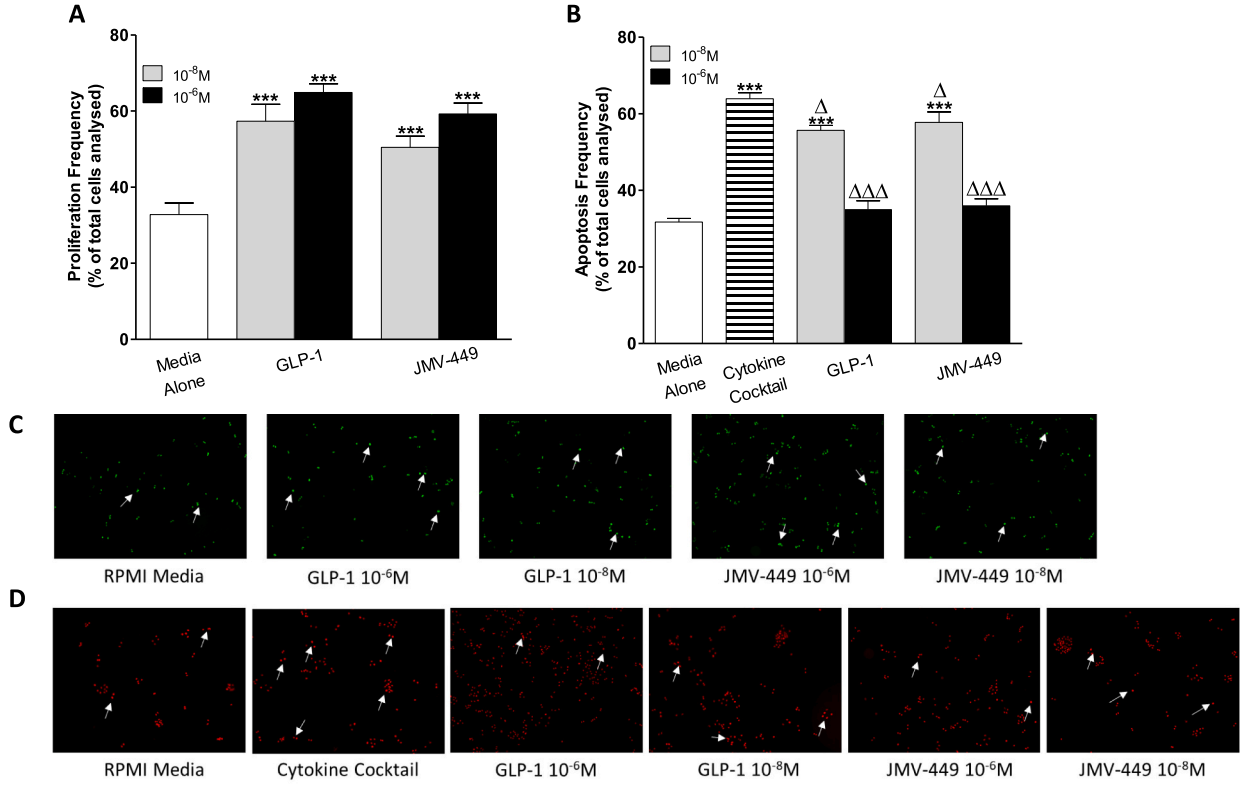

Fig. 2. Effect of JMV-449 on beta-cell proliferation and protection against apoptosis.
BRIN-BD11 cells were incubated overnight (18 h) with GLP-1 or JMV-449 (each at 10^{-8} and 10^{-6} M). (A) Proliferation was measured using Ki-67 immunocytochemistry. (B) TUNEL positive apoptotic cells were assessed following 2 h exposure to a cytokine cocktail (IL-1β 100 U/mL, IFN-γ 20 U/mL, TNF-α 200 U/mL) with or without co-culture in the presence of GLP-1 or JMV-449 (each at 10^{-8} and 10^{-6} M). (C,D) Representative images showing the effects of JMV-449 on proliferation (C, green) and apoptosis (D, red) at 10^{-6} and 10^{-8} M in BRIN-BD11 cells. All images shown are X10 magnification. Arrows indicate proliferating (C) or apoptotic (D) cells. Values represent means ± SEM ($n = 4$). (A) ***P < 0.001 compared to respective media control. (B) ***P < 0.001 compared to respective media control. $^{\Delta}$P < 0.05 and $^{\Delta\Delta\Delta}$P < 0.001 compared to cytokine cocktail.

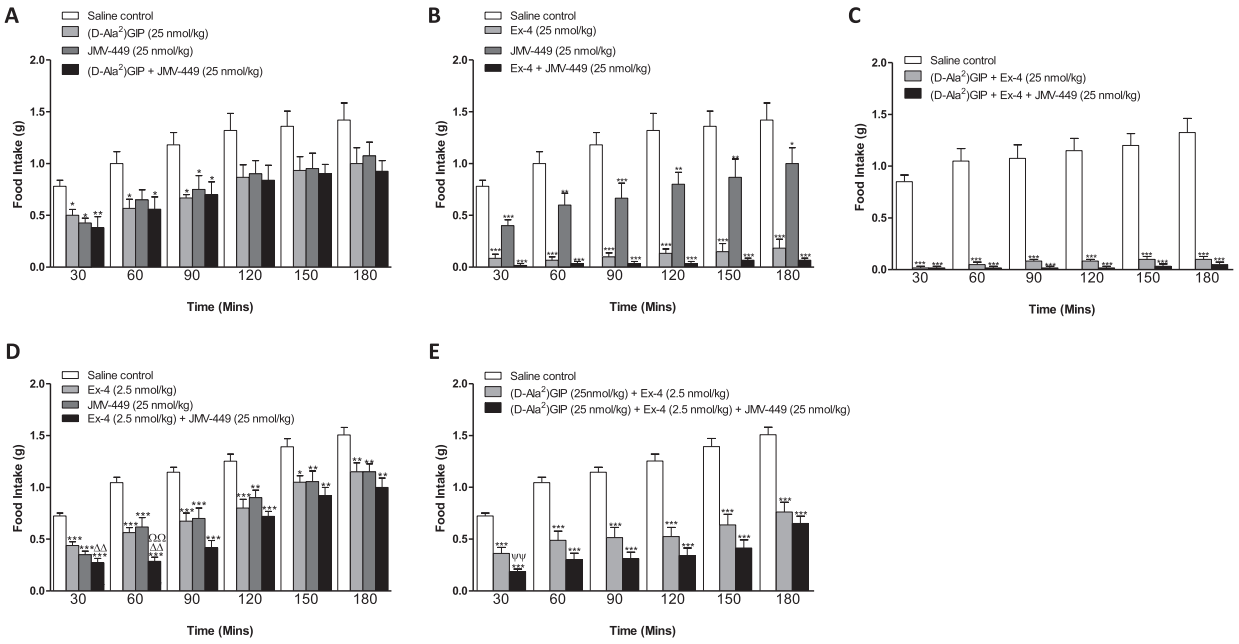

Fig. 3. Effects of JMV-449 alone, and in combination with (D-Ala^2)GIP or exendin-4, on cumulative food intake in mice.
(A-C) Cumulative food intake was measured prior to ($t = 0$) and 30, 60, 90, 120, 150, 180 min after i.p. injection of saline vehicle (0.9% *w/v* NaCl), JMV-449, (D-Ala^2)GIP or exendin-4 alone, and in combination (each at 25 nmol/kg bw), in 18 h fasted mice. (D,E) Experiments were repeated in an identical manner, but with exendin-4 administered at a dose of 2.5 nmol/kg bw. Values represent means ± SEM (n = 8). *P < 0.05, **P < 0.01 and ***P < 0.001 compared to respective saline control. $^{\Delta\Delta}$P < 0.01 compared to exendin-4 (2.5 nmol/kg). $^{\Omega\Omega}$P < 0.01 compared to JMV-449 (25 nmol/kg). $^{\Psi\Psi}$P < 0.01 compared to (D-Ala^2)GIP (25 nmol/kg) plus exendin-4 (2.5 nmol/kg).

Table 2
Effects of JMV-449 alone, and in combination with (D-Ala^2)GIP or exendin-4, on blood glucose concentrations in mice.

Treatment	Blood glucose value at each time point post-injection (mmol/l)					0–105 min AUC (mmol/l.min)
	0 min	15 min	30 min	60 min	105 min	
Glucose alone	6.3 ± 0.2	13.2 ± 0.8	11.8 ± 0.6	9.4 ± 0.4	8.0 ± 0.6	47.2 ± 2.4
Glucose + JMV-449	6.2 ± 0.1	9.1 ± 0.5**	9.7 ± 0.5*	8.3 ± 0.3	7.2 ± 0.3	40.9 ± 3.4
Glucose + (D-Ala^2)GIP	6.5 ± 0.5	8.5 ± 0.4***	7.3 ± 0.4***	6.7 ± 0.3***	6.1 ± 0.4*	35.0 ± 2.1**
Glucose + JMV-449 + (D-Ala^2)GIP	6.3 ± 0.3	7.9 ± 0.7***	6.4 ± 0.3***	6.5 ± 0.2***	6.1 ± 0.2*	26.4 ± 1.2***, $^{\Delta\Delta,\Omega}$
Glucose + exendin-4	6.5 ± 0.4	9.1 ± 0.5**	5.4 ± 0.4***	5.1 ± 0.3***	4.7 ± 0.4***	36.8 ± 2.6*
Glucose + JMV-449 + exendin-4	5.9 ± 0.2	7.6 ± 0.4***	4.9 ± 0.3***	4.3 ± 0.2***	4.4 ± 0.4***	27.5 ± 3.1**,$^{\Delta,\pi}$
Glucose + (D-Ala^2)GIP + exendin-4	6.6 ± 0.4	9.2 ± 0.4**	6.4 ± 0.4***	5.6 ± 0.3***	4.5 ± 0.2***	32.6 ± 1.3**
Glucose + JMV-449 + (D-Ala^2)GIP + exendin-4	6.2 ± 0.3	7.9 ± 0.4***	6.6 ± 0.3***	5.8 ± 0.4***	4.8 ± 0.2***	25.6 ± 1.9***,$^{\Delta\Delta}$

Blood glucose concentrations were measured immediately before and 15, 30, 60 and 105 min after i.p. injection of glucose alone (18 mmol/kg bw) and test peptides (each at 25 nmol/kg bw) in 18 h fasted mice. Glucose AUC values for 0–105 min post injection are also presented. Values represent mean ± SEM for 6 mice. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared to glucose alone. $^{\Delta}$P < 0.05 and $^{\Delta\Delta}$P < 0.01 compared to JMV-449 alone. $^{\Omega}$P < 0.05 compared to (D-Ala^2)GIP. $^{\pi}$P < 0.05 compared to exendin-4.

Table 3
Effects of JMV-449 alone, and in combination with (D-Ala^2)GIP or exendin-4, on plasma insulin concentrations in mice.

Treatment	Plasma insulin value at each time point post-injection (ng/mL)					0–105 min AUC (ng/mL.min)
	0 min	15 min	30 min	60 min	105 min	
Glucose alone	9.9 ± 0.9	13.1 ± 1.0	10.7 ± 0.9	10.8 ± 0.3	10.5 ± 0.8	47.6 ± 3.8
Glucose + JMV-449	11.6 ± 0.6	16.1 ± 1.9	14.0 ± 0.7	12.1 ± 1.2	11.3 ± 0.8	63.0 ± 2.5*
Glucose + (D-Ala^2)GIP	10.9 ± 0.4	13.0 ± 0.8	15.4 ± 0.9*	12.9 ± 0.6	12.1 ± 1.0	61.9 ± 2.2*
Glucose + JMV-449 + (D-Ala^2)GIP	11.1 ± 1.0	16.5 ± 2.5	13.4 ± 1.3	13.2 ± 0.4	12.0 ± 0.6	66.1 ± 4.1**
Glucose + exendin-4	12.1 ± 0.9	16.0 ± 1.3	13.7 ± 1.8	11.4 ± 0.7	11.3 ± 2.3	54.5 ± 5.1
Glucose + JMV-449 + exendin-4	10.9 ± 0.2	14.5 ± 1.5	13.6 ± 1.5	12.0 ± 1.1	11.2 ± 0.8	56.7 ± 1.9
Glucose + (D-Ala^2)GIP + exendin-4	11.2 ± 0.8	14.2 ± 0.6	13.0 ± 0.8	11.4 ± 1.9	10.4 ± 0.5	48.8 ± 4.5
Glucose + JMV-449 + (D-Ala^2)GIP + exendin-4	11.2 ± 0.7	14.6 ± 2.5	12.6 ± 0.4	11.5 ± 0.8	11.3 ± 0.3	56.5 ± 2.7

Plasma insulin concentrations were measured immediately before and 15, 30, 60 and 105 min after i.p. injection of glucose alone (18 mmol/kg bw) and test peptides (each at 25 nmol/kg bw) in 18 h fasted mice. Plasma insulin AUC values for 0–105 min post injection are also presented. Values represent mean ± SEM for 6 mice. *$P < 0.05$ and **$P < 0.01$ compared to glucose alone.

GIP and GLP-1 can be improved by concurrent activation of neurotensin receptors [3,21]. Given that the highly prominent benefits of upregulated incretin receptor activation in various rodent models of obesity-

related diabetes have not been fully translated to the clinic, this is of significant therapeutic interest. In that regard, bariatric surgery appears to be the only intervention that leads to sustained weight loss and potential remission of type 2 diabetes in humans [22], although recent observations using once-weekly subcutaneous injection of the GLP-1 mimetic semaglutide in obese men does offer some encouragement [23]. Interestingly, the most effective forms of bariatric surgery are associated with dramatic changes in the secretion and action of various gut-derived hormones, which is believed to play a major role in promotion of almost immediate resolution of diabetes post-surgery [24]. Several studies demonstrate increased circulating neurotensin concentrations following bariatric surgery in both rodents and humans [3,12,25,26], with surgical-induced modulation of GIP and GLP-1 levels already well accepted [24]. Thus, interplay between incretin and neurotensin receptor signalling likely has an appreciable contribution to the overall observed benefits of bariatric surgery. However, it should also be acknowledged that very low calorie diets do not alter circulating incretin hormone levels but can result in a relatively rapid remission of type 2 diabetes [27], although prolonged adherence to such strict diets appears to be particularly challenging [28].

As expected, JMV-449 was completely resistant to plasma enzymatic degradation, likely as a consequence of the reduced peptide bond present between Lys^8 and Lys^9 [10]. In keeping with the biological actions and insulin secretory profile of neurotensin receptor activation [29], JMV-449 evoked clear dose-dependent increases of insulin secretion from rodent BRIN-BD11 cells at basal glucose concentrations, which were less evident under hyperglycaemic conditions. Thus, this slight variability of insulin secretory profile is in complete harmony with established bioactivity of neurotensin-like peptides on pancreatic beta-cell function [29]. Risk of hypoglycaemia with JMV-449 would be unlikely given that activation of neurotensin receptors is known to induce glucagon secretion at low glucose concentrations [30], but this would need to be confirmed for JMV-449. Moreover, JMV-449 imparted independent benefits on beta-cell growth and survival [31], with potential translational benefits in terms of restoration of beta-cell mass and function in diabetes that require further study. These positive effects on pancreatic beta-cells are very reminiscent of the actions of both incretin hormones, GIP and GLP-1 [32]. Indeed, similar to observations with GIP and GLP-1 [33], neurotensin, and the related hormone xenin that likely functions in part as a neurotensin receptor agonist [8], are evidenced locally within the pancreas and believed to impart important autocrine or paracrine effects on islet function [31]. As such, we observed clear potentiating actions of JMV-449 on GIP- and GLP-1-induced insulin secretion at basal glucose concentrations, with similar effects previously reported following combined treatment of GIP with xenin [4–7,34–36]. Indeed, these positive GIP/xenin interactions ultimately led to development of a unimolecular dual-acting GIP/xenin hybrid peptide [37], which displays significant and sustained antidiabetic effects in rodent models of diabetes [38]. However, it should be noted that the incretin hormone potentiating actions of JMV-449 were much less obvious at elevated 16.7 mM glucose concentrations in the current setting.

In full harmony with *in vitro* findings, JMV-449 possessed prominent glucose homeostatic actions in lean healthy mice that were directly linked to elevated plasma insulin concentrations. Importantly, positive additive benefits were noted in terms of glycaemic status when JMV-449 was administered together with stable GIP or GLP-1 mimetics, although this was not associated with augmentation of glucose-induced insulin secretion. This is interesting, and could relate to reduced insulinotropic effectiveness of neurotensin at elevated glucose levels [29]. However, additive benefits of neurotensin and incretin hormones to improve glucose disposal could point towards insulin-independent glucose-lowering actions. In this respect, GIP, GLP-1 and neurotensin all exert established, but somewhat distinctive, effects on glucagon secretion [12,39,40], which could be a factor here. In addition, upregulated incretin hormone signalling exerts well described extrapancreatic glucose-lowering actions [39,41], which may be augmented by neurotensin signalling. Both avenues are complex and as such require additional detailed study that is outside the scope of the current investigation. For example, recent studies with GIP and xenin suggest that neurotensin receptor activation can partially reverse the glucose-dependent glucagonotropic action of GIP [6], whereas the impact of neurotensin on the glucagonostatic action of GLP-1 is unknown.

Neurotensin receptor activation exerts well documented effects to suppress appetite [42], with similar actions noted with GLP-1 [43] and more recently GIP [44]. These combined actions would be highly favourable in obesity-driven forms of diabetes, such as type 2 diabetes mellitus. Our studies confirmed inhibitory effects on food intake following individual administration of JMV-449, (D-Ala^2)GIP or exendin-4 at a dose of 25 nmol/kg. Receptors for GIP, GLP-1 and neurotensin are located within the paraventricular nuclei region of the hypothalamus, known to critical for energy regulation and metabolism [45–47], and likely mediates these effects. The excellent efficacy of exendin-4 alone at a dose of 25 nmol/kg precluded any conceivable additive appetite suppressive effects. However, with a reduced dose of exendin-4 there was good evidence for JMV-449 additive benefits on appetite reduction in mice. As such, it has been suggested that sub-threshold doses of GLP-1 are required to induced appetite and weight loss synergy with neurotensin [3]. JMV-449 was also unable to enhance (D-Ala^2)GIP mediated reductions in food intake, which may be peptide dose and model related. However, studies with GIP and xenin in rodents also suggest that unlike effects of glucose homeostasis and insulin secretion, these peptides lack additive positive actions on appetite control [21].

Although potential benefits of combined incretin and neurotensin signalling have been previously suggested [3,5], this is the first study to directly evaluate possible interplay between both incretin hormones and neurotensin. It is reassuring to note that JMV-449 evoked clear *in vitro* insulinotropic and *in vivo* glucose-lowering potentiating benefits in combination with GIP and GLP-1, suggesting no obvious threshold for these benefits. Appropriate dose adjustment within the *in vivo* series of experiments may have allowed for observation of further additive benefits. With a view towards possible clinical application of our findings, the short sequence of JMV-449 is highly attractive, making it cheap and relatively easy to synthesise, as well as potentially allowing a non-injectable peptide drug administration [48]. In that regard, recent approval of oral delivery of the GLP-1 mimetic semaglutide [49], as well as confirmed oral bioavailability for DPP-4 inhibitor drugs [48], is encouraging. Moreover, advances in regulatory peptide drug design and development, leading to creation of functional unimolecular triple agonists, represents another potential route to clinical realisation of the benefits of combined GIP, GLP-1 and neurotensin receptor signalling.

In conclusion, these data establish that JMV-449 is an enzymatically stable and bioactive hexapeptide analogue of neurotensin, which possesses notable positive effects on pancreatic beta-cells. Furthermore, we clearly demonstrate the ability of JMV-449 to significantly potentiate the glucose homeostatic and insulin releasing actions of the incretin hormones, GIP and GLP-1. Further consideration is required to fully assess the clinical potential of upregulated neurotensin signalling in combination with approved incretin-based therapeutics.

Author contributions

NI, VAG and GH conceived/designed the study. NI and SLC drafted the manuscript. SLC and CES participated in the conduct/data collection and analysis and interpretation of data. All authors revised the manuscript critically for intellectual content and approved the final version of the manuscript.

Declaration of Competing Interest

All authors declare no conflict of interest.

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